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Title: **Phosphate availability affects the tonoplast localization of PLD ζ 2, an *Arabidopsis thaliana* phospholipase D**

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Abbreviations:

PC, phosphatidylcholine; PE, phosphatidylethanolamine; DGDG, digalactocyldiacylglycerol; MGDG, monogalactocyldiacylglycerol; Pi, phosphate; GFP, green fluorescent protein; PX, phox homology domain; PH, pleckstrin homology domain.

1. Introduction

In *Arabidopsis thaliana*, the phospholipase D, PLD ζ 2, has been shown to play a role in lipid modification induced by Pi deprivation [1,2]. When submitted to Pi deprivation, plants change their lipid composition and recycle Pi from phospholipids [3,4]. Phospholipids, especially PC and PE, decrease and the DGDG galactolipid increases. The induced DGDG accumulates not only in plastids where it is usually confined but also in extra-plastidic membranes such as mitochondria, plasma membrane and tonoplast [5-7]. Some genes involved in this induced galactolipid synthesis, *mgd2/3* and *dgd2* are overexpressed [8-10]. A strong and rapid overexpression of PLD ζ 2 was also observed [1,2,10]. Since under Pi deprivation, the absence of PLD ζ 2 reduces the capacity for galactolipid formation from the DAG moieties of phospholipids, it has been proposed that PLD ζ 2 plays some role in the lipid transition possibly by feeding with PA the galactolipid synthesis that occurs in the plastid envelope [1,2]. Additionally, reports provided evidence that PLD ζ 2 is involved in root system architecture [1,11,12], auxin-dependent hypocotyl elongation and vesicle cycling [11]. Whether all these described phenotypes are directly mediated by PLD ζ 2 and how these diverse roles of PLD ζ 2 are connected remains to be established. For the understanding of the multiple functions described for PLD ζ 2, localization of the protein appears as a prerequisite. However it is still unknown. In this report, we demonstrate and analyze the tonoplast localization of PLD ζ 2.

2. Materials and methods

Plant materials and growth conditions

Photosynthetic *Arabidopsis thaliana* cell suspension was cultured as in [4]. *Arabidopsis* plants were cultured on Murashige and Skoog medium (Duchefa) with 0.5 % sucrose with 1.25 mM Pi (+Pi) or with 5 μ M Pi (-Pi) under a daily 10-hour light cycle (150 μ mol photons m⁻² s⁻¹).

PLD ζ 2-GFP fusion construct

Full length PLD ζ 2 cDNA was obtained from Drs M. Seki and K. Shinozaki (RIKEN, Japan). Frame shift at position nucleotide 1712 from start of the coding sequence of At3g05630 was corrected by PCR as indicated in (Suppl Material and methods). PCR products were sequenced and digested by *Sal*I and *Nco*I restriction enzymes. Digested fragments were fused upstream and inframe with *GFP S65T* gene under the control of the CaMV 35S promoter in pUC 18.

Plant transformants

Biolistic transformation of 10 d-old pea leaves was done as in [13]. DsRed and KCO1::DsRed vectors were kind gifts of S. Thomine and K. Czempinski. PLD ζ 2-GFP cDNAs were inserted into the binary vector, pEL103, and into *Arabidopsis* Col via *Agrobacterium* using flower dip method. The *pld ζ 2* KO mutant is the *Arabidopsis* homozygous insertion mutant SALK_094369 described in [1,12]. *CaMV 35S-NRAMP3-GFP* and *CaMV 35S-TIP1;1-GFP Arabidopsis* lines are described in [14,15].

Extraction of tonoplast enriched fraction

Membrane fractions were prepared as in [16] except that cells or plants were ground as in [7]. Tonoplast-enriched fraction was recovered from the top of the sucrose gradient and heavy membrane-enriched fraction was collected at the interface between the 25 %-30 % sucrose layers.

Immunodetection analysis

PLD ζ 2 antibodies were obtained by rabbit immunization with a fusion protein containing the Nter 16 amino acid sequence of PLD ζ 2 linked in Cter to ovalbumin by a cystein and purified by affinity on the corresponding PLD ζ 2 oligopeptide. E37, γ -TIP, V-ATPase antibodies were used as in [7,14]. 2E7 and W1C antibodies (gifts from B Satiat-Jeunemaitre and M Boutry) were used at 1/100 and 1/1000 dilution. Monoclonal of Anti-GFP (Clontech) was used at 1/2000 dilution. Immunodetection was done as described in [7].

Confocal microscopy

Confocal imaging was done as in [7]. Each fluorescence emission was imaged sequentially (400 Hz, line to line) to avoid signal overlapping. GFP fluorescence emission was measured between 500-539 nm with excitation at 488 nm, DsRed fluorescence between 555-616 nm with excitation at 543 nm; chlorophyll fluorescence between 644-726 nm with excitation at 633 nm, mitotracker orange fluorescence between 555-616 nm with excitation at 543 nm.

3. Results

PLD ζ 2 localizes to tonoplast

PLD ζ 2 protein has been detected as a possible vacuole protein by a single proteomic analysis [16]. To verify this localization, we performed several assays of vacuole fractionation from *Arabidopsis* cells or plants. A protein was immunodetected at the expected size for PLD ζ 2 (118 kDa) in the microsome fraction of *Arabidopsis* cells (Fig.1A). This signal was weak and increased in a tonoplast-enriched fraction correlated with tonoplast markers such as γ -TIP (tonoplast intrinsic protein) and V-ATPase. In contrast, it did not show any correlation with chloroplast envelope, ER or plasma membrane markers. Under Pi deprivation, the signal was stronger as expected from enhanced *pld* ζ 2 expression and still enriched by purification of the tonoplast fraction (Fig.1B). A protein was also immunodetected in a tonoplast fraction similarly prepared from *Arabidopsis* plant leaves and was not present in a *pld* ζ 2⁻ KO mutant confirming the identity of the PLD ζ 2 signal (Fig.1C). Several smaller proteins were immunodetected only in the wild type plant suggesting some PLD ζ 2 degradation. Altogether, immunodetection experiments indicated an association of PLD ζ 2 with the tonoplast.

To find out the PLD ζ 2 localization *in vivo*, we prepared a set of expression constructs of green fluorescent protein (GFP) fused to the C-terminus of either the entire PLD ζ 2 protein (PLD ζ 2::GFP) or the putative regulatory domain of PLD ζ 2 excluding the phospholipase activity domain [17] (Nter-PLD ζ 2::GFP) (Suppl Fig. 1). The fusion proteins were transiently expressed in pea leaves by biolistic transformation. By confocal imaging after expression of PLD ζ 2::GFP, we observed GFP fluorescence at the tonoplast in different types of leaf cells e.g. stomata guard cells and epidermal cells (Fig. 2). An additional but weak fluorescence was sometimes detected in the cytosol. For a better delineation of tonoplast, DsRed was co-introduced as a soluble cytosolic and nuclear control. GFP fluorescence was thus detected along the DsRed fluorescence at expected position for tonoplast. When PLD ζ 2::GFP was co-introduced with a tonoplast marker KCO1::DsRed, PLD ζ 2::GFP, fluorescence co-localized with KCO1::DsRed fluorescence. These results altogether demonstrated a localization of PLD ζ 2 to the tonoplast. Stronger GFP signal and similar sorting to the tonoplast were obtained with Nter-PLD ζ 2::GFP indicating that the Nter part of PLD ζ 2 was sufficient to address the PLD ζ 2 protein to the tonoplast and suggesting that expression of the GFP fusion protein was stabilized in absence of phospholipase activity domain.

Proliferation of membranes inside the vacuole of the *pld* ζ 2⁻ KO mutant

Immunodetection and confocal imaging indicated the localization of PLD ζ 2 in the tonoplast. The presence of PLD ζ 2 in the tonoplast membrane can be important for this membrane. We checked the aspect of the tonoplast in the *pld ζ 2*⁻ KO mutant. By electron microscopy, we observed an unusual abundance of membranes inside the vacuole (Fig. 3). This result was confirmed by imaging of the NRAMP3::GFP tonoplast marker. After biolistic transformation of 30 day old plant leaves, the GFP fluorescence indicated membrane budding inside the vacuole of the *pld ζ 2*⁻ KO mutant that was not apparent in the wild type (Fig. 3 and Suppl Fig. 2). Electron microscopy and confocal imaging therefore indicated that, in absence of PLD ζ 2, vacuole membrane development was different.

PLD ζ 2 modifies its distribution on the tonoplast in the Pi deprived condition

Under Pi deprivation, the suppression of PLD ζ 2 reduces the lipid modification associated with Pi-deprivation [1,2]. In order to analyze if the localization of PLD ζ 2 was affected by Pi deprivation, *Arabidopsis* was stably transformed via *Agrobacterium* transfection with binary vectors containing PLD ζ 2::GFP or N-ter-PLD ζ 2::GFP downstream of the CaMV35S promoter. Both in *PLD ζ 2-GFP* and *Nter-PLD ζ 2-GFP* plants, GFP fluorescence was located at the tonoplast as observed in pea. Relative position of mitochondria and chloroplasts as determined respectively by mitotracker and chlorophyll fluorescence confirmed localization to tonoplast (Suppl Fig. 3). Under Pi deprivation, GFP fluorescence was still located at the tonoplast in the *PLD ζ 2-GFP* plants (Supl Fig.4) as indicated by cell fractionation analysis. The same localization was observed with Nter-PLD ζ 2::GFP (Fig. 4). The overall aspect of the GFP fluorescence was however different from the Pi sufficient condition. The distribution along the membrane was rather uneven especially in young seedlings. When the *Nter-PLD ζ 2-GFP* plants were grown on -Pi medium for 11 days, GFP fluorescence formed a dot pattern on tonoplast (Fig.4). This dot pattern was also observed in the control plants grown on +Pi medium but the number of highly fluorescent dots in the -Pi condition was 4-5 times higher than in the +Pi condition. After 16 days of culture, the dots were brought back to low level in both conditions. We verified that level of GFP protein was similar in all conditions (Fig. 4). The punctuate pattern of the tonoplast was moreover specific to (Nter) PLD ζ 2 since we did not observed it with 2 other tonoplast GFP markers (Fig 5). This therefore indicated transient formation of tonoplast domains enriched in PLD ζ 2 and some role of the N-ter part of the protein in this formation.

Since DGDG synthesis induced by Pi deprivation is located in the plastid envelope and since mitochondria interact with plastids at an early stage of Pi deprivation [7], we analyzed the possible interaction of these domains with chloroplasts and mitochondria. By chemical labeling with mitotracker, we observed in root cells that most of GFP-fluorescent domains were very close to mitochondria (about 80 %) (Fig. 6 and suppl Table I). In the leaf tissue, mitotracker labeling was difficult to obtain under Pi deprivation. We however obtained some images showing that GFP domains were close to mitochondria too. We also observed that a relatively high proportion of GFP-domains were beside chloroplasts (34%) (Fig. 5 and 6 and suppl Table I).

4. Discussion

In this report, we demonstrated by cell fractionation and confocal imaging on plants that PLD ζ 2 localizes to the tonoplast. The Nter part of PLD ζ 2 was sufficient to address PLD ζ 2 to the tonoplast. Pi deprivation did not change PLD ζ 2 addressing to tonoplast. It however enhanced transient formation in the tonoplast of PLD ζ 2-enriched domains, which were preferentially positioned very close to mitochondria and beside chloroplasts.

How PLD ζ 2 is recruited to tonoplast

Bioinformatic analyses of the PLD ζ 2 protein sequence predicted a cytoplasmic localization of PLD ζ 2 without any define sorting signal. Since in our experiments both PLD ζ 2::GFP and Nter-PLD ζ 2::GFP localized to tonoplast, it indicates that the tonoplast addressing information is present in the Nter regulatory domain of the protein. In this domain, there are two putative membrane-interacting domains, PH and PX domains, which can play some role in the recruiting. Among *Arabidopsis* PLDs, PLD ζ 1 and PLD ζ 2 are characterized by the presence of these 2 domains. They share this specific feature with mammalian PLDs, i.e. PLD1 and PLD2 [17]. For mammalian PLDs, both PH and PX are essential domains for their localization and regulation [18]. PLD1 localizes to perinuclear region, ER and golgi apparatus, and PLD2 localizes to plasma membrane. The deficiency of PH domain in PLD2 induces the mislocalization of the protein to endosome [18]. PH and PX domains in the mammalian PLD bind to phosphoinositides, such as PI(3,4,5)P3 and PI(3)P [18]. This interaction with lipids determines the localization of PLD. Therefore, it is possible that PLD ζ 2 is addressed to tonoplast through interaction of PX and PH domains with specific lipids without excluding possible necessity of interaction with particular proteins.

Localization of PLD ζ 2 and the role of the protein

The intracellular localization of PLD ζ 2 to tonoplast should help to infer its role. In standard conditions, PLD ζ 2 is expressed although at reduced level in leaves and in roots and it was shown to participate to auxin-dependent response through vesicle recycling of PIN2 auxin receptor [11]. In our study, we observed that vacuolar membrane development was stimulated in the absence of PLD ζ 2. Therefore, it suggests that PLD ζ 2 has also a role in vacuolar membrane dynamics.

Under Pi deprivation, PLD ζ 2 enhances the capacity for galactolipid formation from the DAG moieties of phospholipids. Tonoplast as well as mitochondria lipid composition are modified with a decrease of phospholipids and an increase in DGDG [5]. The localization of PLD ζ 2 in tonoplast likely indicates that the protein is active on tonoplast phospholipids. However the close proximity of PLD ζ 2-enriched domains with mitochondria can also indicate that PLD ζ 2 interacts with the lipid modification that occurs in mitochondria. As a support for this hypothesis, the increased number of contact sites between mitochondria and chloroplasts that we observed on cell culture at an early stage of Pi deprivation [7] can possibly be related to the transient increase of PLD ζ 2-enriched tonoplast domains observed in the present work at an early stage of plant growth under Pi deprivation. The position of PLD ζ 2 domains beside chloroplasts, where mitochondria often position (see fig 3D), further sustains this hypothesis. High concentration of PLD ζ 2 could facilitate transfer of lipids between mitochondria and chloroplasts.

Membrane lipid alteration during Pi starvation was shown to be dependent on both Pi signaling and auxin/cytokinin cross-talk [19]. Since soluble Pi is mainly stored in the vacuole, and since Pi signaling is likely connected to Pi storage, it will be interesting to analyze if tonoplast localization of PLD ζ 2 enables some function of PLD ζ 2 in vacuolar Pi storage.

5. Figure legends:

Fig.1: Western blot immunodetection of PLD ζ 2 protein in *Arabidopsis* fractions. A: Comparative detection in *Arabidopsis* cell fractions. M: crude microsome fraction, T: tonoplast fraction, HM: heavy membrane-enriched microsome fraction, CE: chloroplast envelope fraction. γ -TIP and V-ATPase: tonoplast markers; E37: a plastid envelope marker, 2E7: a ER marker and W1C: a plasma membrane marker. B: PLD ζ 2 detection in microsomes

and tonoplast fraction of *Arabidopsis* cells grown with 1.25 mM Pi or without Pi. C: PLD ζ 2 detection in tonoplast fraction of *Arabidopsis* wild-type or *pld ζ 2* KO mutant plant. Loading of the two lanes are controlled by Ponceau staining on the left and V-ATPase immunodetection underneath.

Fig.2: Confocal observation in guard and epidermal cells after biolistic transformation of pea leaves with PLD ζ 2::GFP or Nter-PLD ζ 2::GFP and DsRed as a control for cytosol and nucleus localization or KCO1::DsRed as a control of tonoplast localization. A to E: GFP fluorescence. F to J: DsRed fluorescence. K to O: Merged images of GFP (green) and DsRed (red) fluorescence. Arrows indicate some typical features of tonoplast delineation. Scale bar: 10 μ m.

Fig.3: Observation of vacuole in *pld ζ 2* KO mutant and in wild-type *Arabidopsis* plant. A to D: Electron microscopy observation in cotyledons of 14 day old plants. EM was done as in [7]. Arrows indicate membrane structures inside the vacuole of the *pld ζ 2* KO mutant. E and F: Confocal imaging of a tonoplast marker NRAMP3::GFP after biolistic transformation of leaves of 30 day old *Arabidopsis* plants grown on soil, *pld ζ 2* KO mutant (E) and wild-type (F).

Fig.4: Compared confocal observations in guard cells of *Nter-PLD ζ 2-GFP Arabidopsis* plants grown with 1.25 mM (A and D) or 5 μ M Pi (B and E) for 11 or 16 days. A, B, D and E: GFP fluorescence. C and F: Average number of GFP dots per cell ($n \geq 10$ cells). G: Immunodetection of Nter-PLD ζ 2::GFP by Western blot using anti-GFP antibodies in the different conditions.

Fig.5: Confocal observations in guard cells of *Nter-PLD ζ 2-GFP*, *TIP-GFP* and *NRAMP3-GFP Arabidopsis* plants grown 11 days with 5 μ M Pi. A, B and C show superposed images of chlorophyll fluorescence in red and GFP fluorescence in green. A strongly punctuated fluorescence pattern of tonoplast was always observed with PLD ζ 2, whereas some delimited areas with enhanced fluorescence were rarely observed with TIP. Overexpression of NRAMP3::GFP led to fluorescent labeling of tonoplast with specific enrichment in some large vesicles engulfed inside the main vacuole. Scale bar: 20 μ m.

Fig.6: Confocal observations of GFP fusions of PLD ζ 2 in *Arabidopsis* plants under Pi deprivation. Localization of GFP dots relative to mitochondria and chloroplasts. A and B: GFP

(green) and mitotracker (red) in *Nter-PLD ζ 2-GFP* root cells. C and D: GFP (green) and mitotracker (red) in leaf cells of *PLD ζ 2-GFP* (C) and of *Nter-PLD ζ 2-GFP* (D). Arrows indicate where GFP dots position close to mitochondria E and F: GFP (green) and chlorophyll (red) in leaf cells of *Nter-PLD ζ 2-GFP Arabidopsis* plants. Scale bar: 5 μ m.

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Figure 1

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Fig. 1

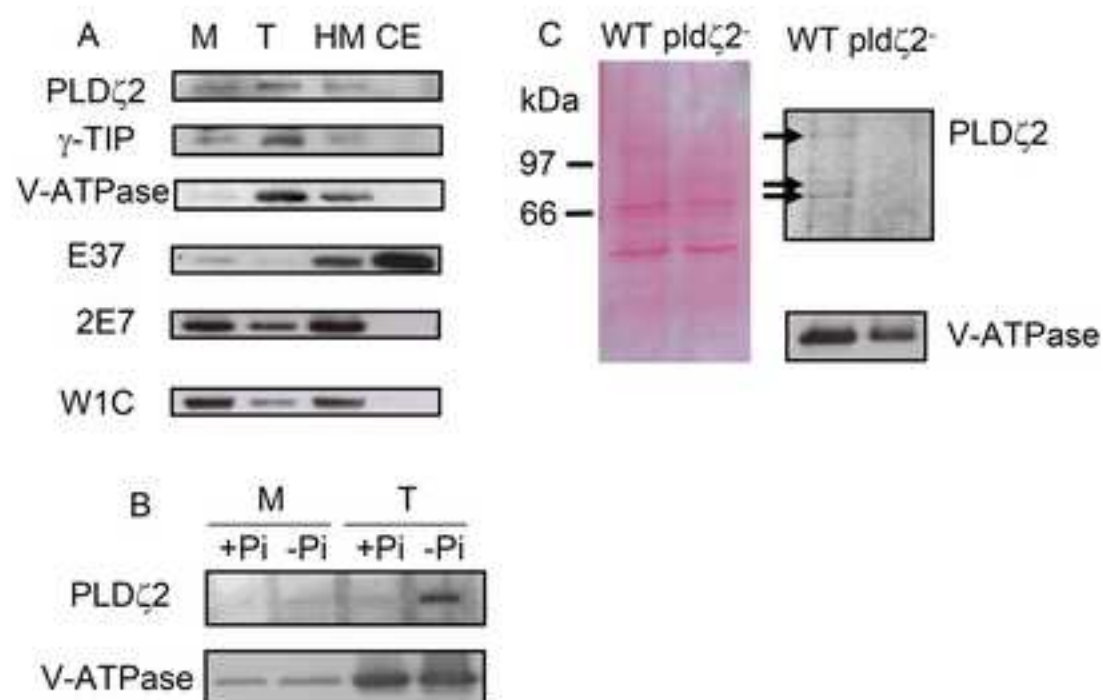


Figure 2

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Fig. 2

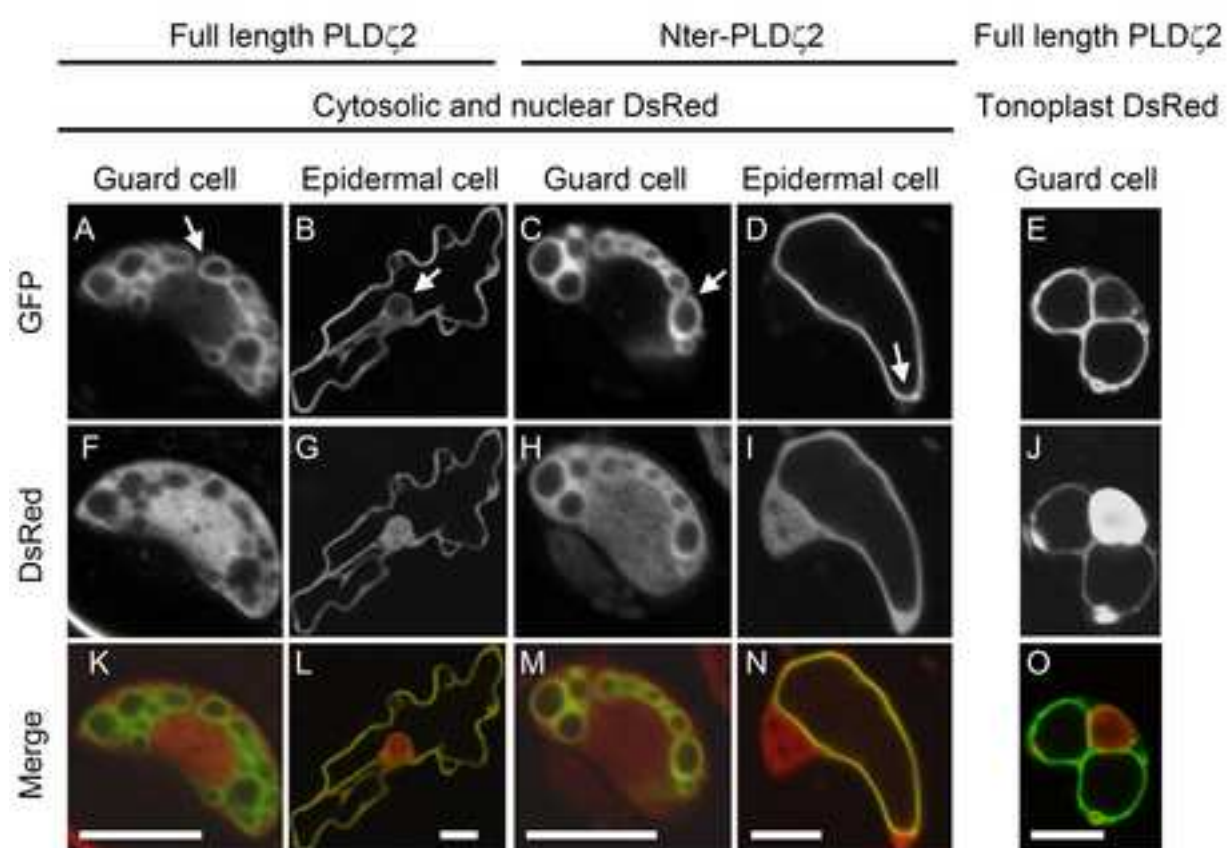


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Fig. 3

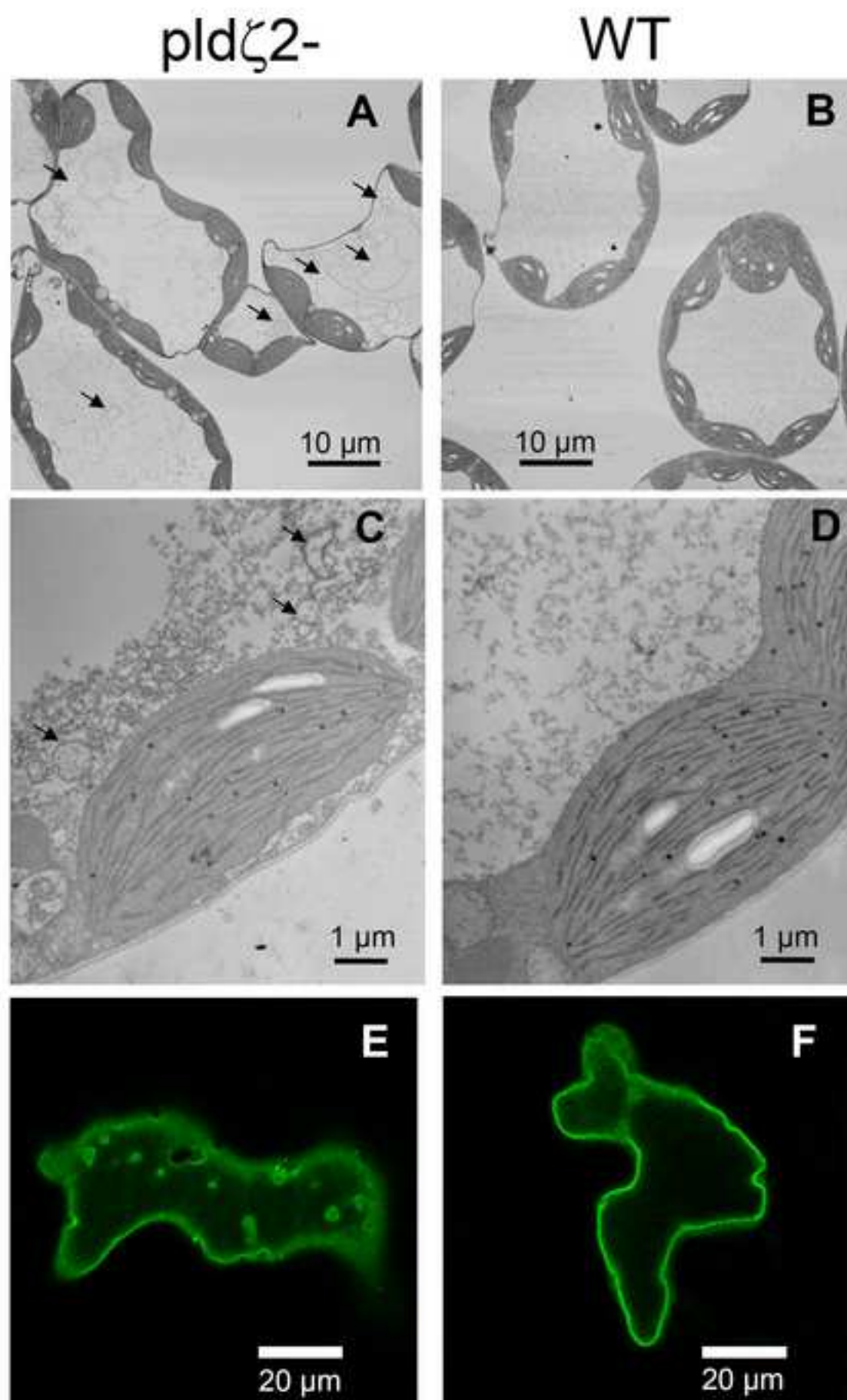


Fig. 4

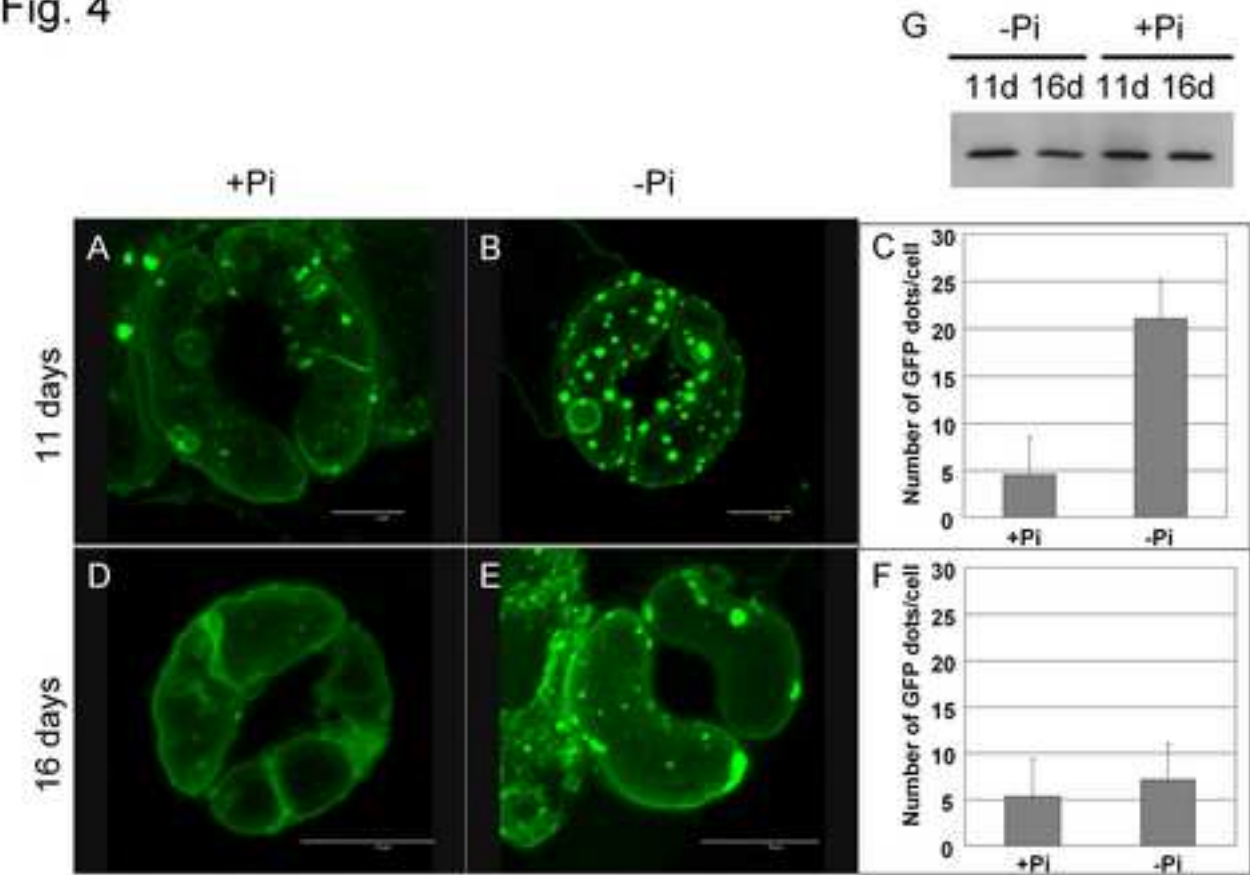


Fig. 5

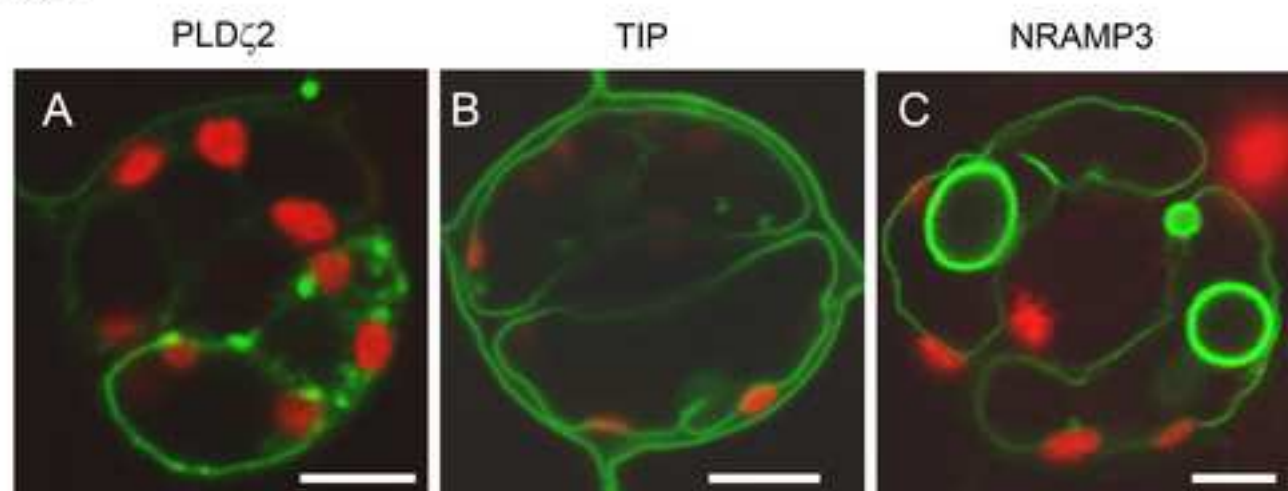
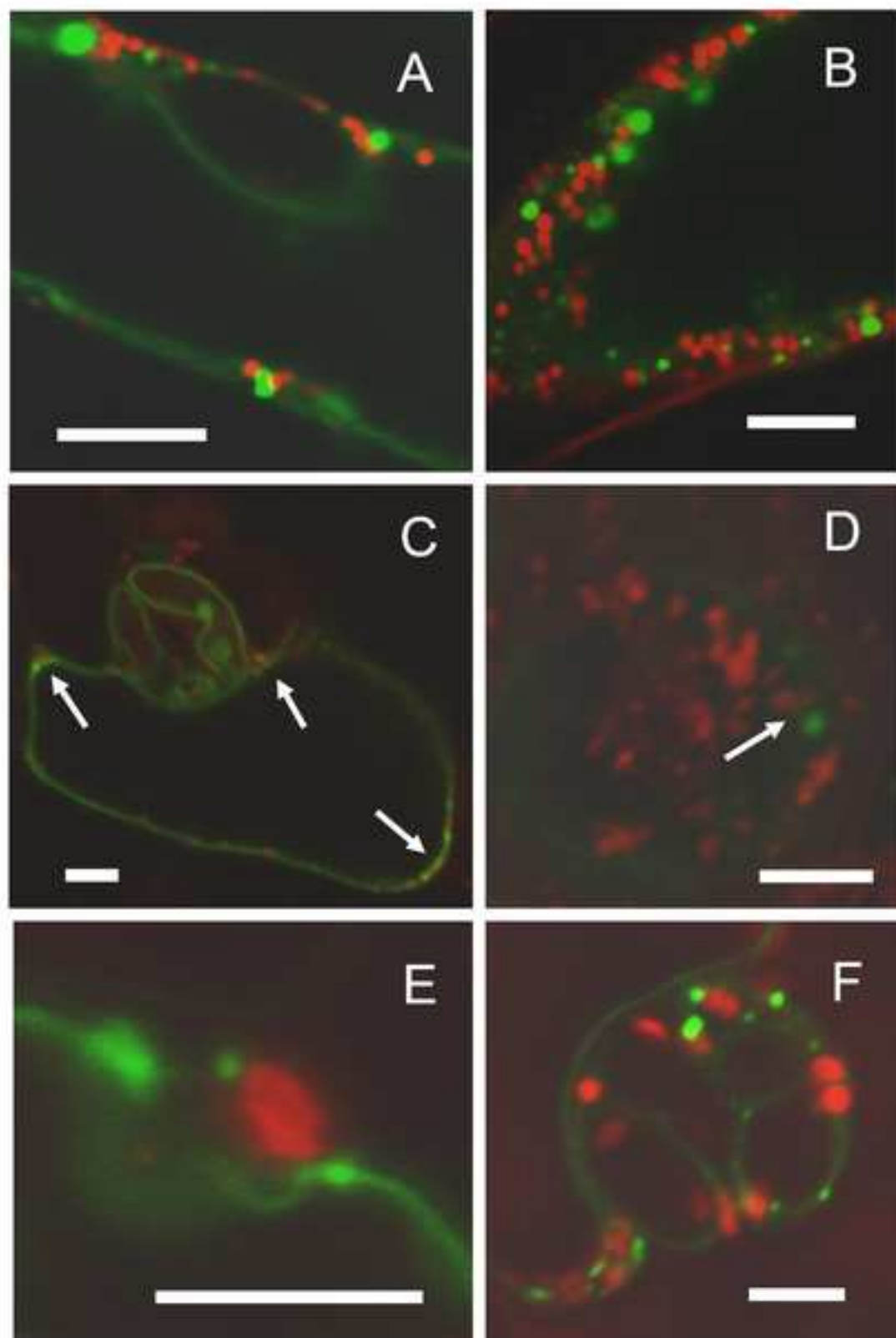


Figure 6

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Fig 6



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